Int. J. Dev. Biol. 37: 125-133 (1993)

Polyomavirus enhancer requirements for expression in embryonal carcinoma cells

LARRY A. COUTURE[1] and JOHN M. LEHMAN*

Department of Microbiology, Immunology and Molecular Genetics, Albany Medical College, Albany, New York, USA

ABSTRACT Wild type polyomavirus expression is suppressed in embryonal carcinoma (EC) cell lines. This suppression is alleviated when the EC cells are induced to differentiate. Several characterized host range mutants of polyoma overcome suppression and are able to express and replicate in the undifferentiated EC cells. These previously described isolates were obtained by serial passage of a wild type strain through PCC4 or F9 EC lines. We present a new pyPCC4 isolate (LPT) derived without selection in EC cells. Isolates with host range specificity for a given EC line have been reported to share several common rearrangements and features. These features are also observed in LPT. We report a novel feature shared by these mutants, including LPT, capable of expression in the EC cell line PCC4. In 8 of 10 isolates a novel sequence is created within the enhancer region by rearrangement junctions with near perfect homology to the AP-1 core consensus sequence, 'TGACT(C/A)A'. That the precise location of these junctions varies among these isolates suggest a functional role for this conserved sequence. Our goal is to understand the function of various mutations in host range mutants of polyoma. In order to understand the rearrangements necessary for expression and replication of polyoma in PCC4 cells, we have further characterized the limits of the B enhancer in these cells as compared to those described in permissive cell systems. We have been able to locate the origin proximal limit of the B enhancer for replication close to nt 5189 and distinguish it from the origin proximal limit of the B enhancer for transcription near nt 5215. The two B enhancer cores overlap but do not coincide and are conserved in both cell lines.

KEY WORDS: *polyoma, embryonal carcinoma, enhancer, stem cells, teratocarcinoma*

Introduction

The murine teratocarcinoma is an interesting tumor that was defined by the work of Dr. G. Barry Pierce and his collaborators. I became interested in this tumor model after reading the papers of Pierce *et al.* regarding the capability of the stem cells of the tumor, embryonal carcinoma, to differentiate into representative cells and tissues of the three embryonic germ layers (Kleinsmith and Pierce, 1964; Pierce, 1967). Following discussion with G. Barry Pierce, we decided to develop an *in vitro* model of the teratocarcinoma and proposed to infect the teratocarcinoma cells with the two papovaviruses, SV40 and polyoma (Lehman *et al.*, 1974; Swartzendruber and Lehman, 1975). Our aim was to determine whether there would be changes in the regulation of the expression of the virus genome in this differentiating cell system. To our surprise when these experiments were performed, we demonstated that the viruses expressed in the differentiated cells but did not express in the embryonal carcinoma cells (synthesis of T antigen). In my laboratory, Drs. Douglas Swartzendruber, Wendell Speers, Thomas Friedrich, Katrina Trevor, Karen Hales, Mary Kay Francis and Larry Couture added to these early studies. Other virologists and cell biologists became interested in the viral-cell model system in attempting to define gene regulation in a differentiating model system at the cellular and viral level. Dr. Pierce always maintained an interest and had significant input into many of these studies using this «interesting» tumor. The studies reported below are a continuation of our interests in defining the regulation of polyoma in this fascinating tumor model system.

The polyoma enhancer region is contained within a 244 bp fragment of non coding DNA adjacent to the viral origin of replication (Devilliers and Schaffner, 1981; Tyndall *et al.*, 1981). This region

Abbreviations used in this paper: EC, embryonal carcinoma; LPT, large plaque Toronto; EOTnt, enhancer region, origin of replication, T antigen coding sequence and origin distal limit of the deletion; T, polyoma tumor antigen; V, polyoma coat protein; ALB, polyoma strain; P16, polyoma strain.

***Address for reprints**: Department of Microbiology, Immunology and Molecular Genetics, A-68, Albany Medical College, Albany, NY 12208, USA. FAX: 518-445.5748.

[1]**Present address:** Genzyme Corporation, 1 Mountain Road, Framingham, MA 01701, USA.

0214-6282/93/$03.00

© UBC Press

Printed in Spain

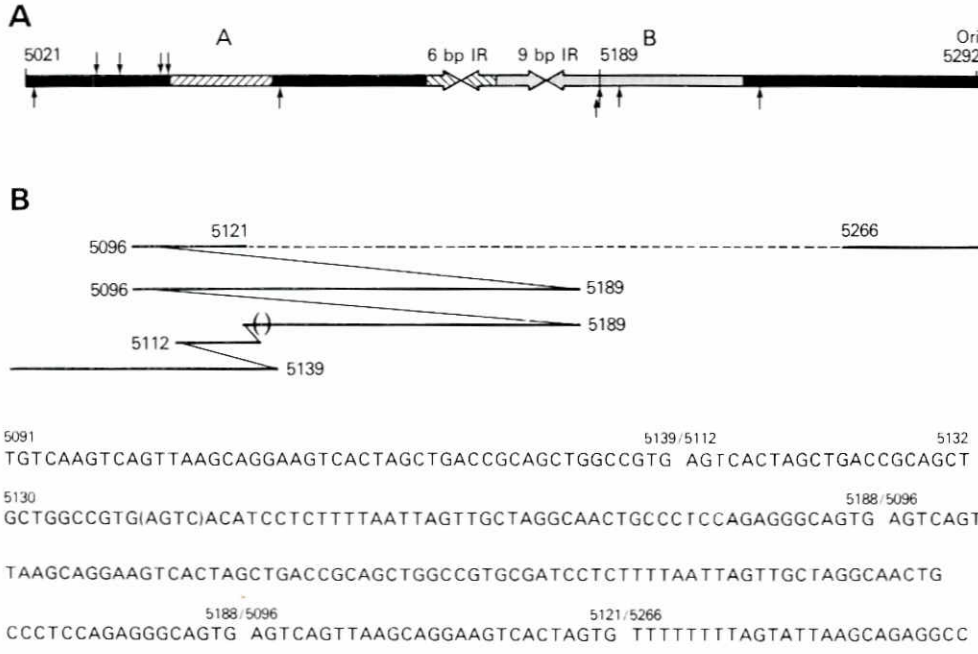

Fig. 1. Sequence analysis of Large Plaque Toronto (LPT). (A) *Representation of the wild type A2 strain showing A and B enhancer cores and inverted repeats. Upper arrows indicate previously described 5' duplication junctions, lower arrows indicate 3' junctions.* **(B)** *Sequence and linear representation of comparable region of LPT showing 3 to 4 copies of the A core, two copies of the inverted repeats and deletion of the B enhancer core. ( ) Represent bases not occurring in A2.*

is required for early and late gene activation and for replication of the virus (Devilliers *et al.*, 1982). Replication and transcriptional control by this region is mediated by two enhancer elements, A (nt 5021 to 5132) and B (nt 5132 to 5269) (Herbomel *et al.*, 1984; Veldman *et al.*, 1985; Muller *et al.*, 1988). The core elements of the A enhancer for replication and transcription overlap and lie between nt 5108-5130 (Veldman *et al.*, 1985). The B enhancer core element for transcription has been defined between nt 5175-5229 (Herbomel *et al.*, 1984) and includes an SV40 core consensus sequence as defined by Weiher (Weiher *et al.*, 1982) and an adjacent 9 bp inverted repeat (IR). The B enhancer core for replication was reported between nt 5168-5202 covering the entire 9bp IR and SV40 homology (Muller *et al.*, 1988).

Embryonal carcinoma (EC) cells are normally refractory to polyoma expression but this block is lost when the cells are induced to differentiate (Swartzendruber and Lehman, 1975). The block to expression occurs after absorption, penetration, uncoating and transport to the nucleus of the viral genome (Swartzendruber *et al.*, 1977) suggesting an inhibition of transcription and/or replication. Several laboratory mutants of polyoma have been identified and characterized with host range specificity for undifferentiated F9 or PCC4 EC cells (termed pyPCC4 and pyF9 isolates) (Vasseur *et al.*, 1980; Fujimura *et al.*, 1981; Katinka *et al.*, 1981; Melin *et al.*, 1985a,b). This altered tropism is conferred by rearrangements of viral sequences encompassing the various polyoma enhancer elements. pyPCC4 host range mutants have several features in common as has been previously described (Melin *et al.*, 1985b). These mutants share a minimal duplication of a sequence that corresponds very closely with the core of the A enhancer. A deletion of variable size and position occurs in all mutants involving sequences within the small 134 bp fragment defined by Pvu II restriction sites at nt 5128 and 5262. Notably, this fragment encompasses the B enhancer element. While no common sequences are deleted, six of seven deletions extend through most of the B enhancer core element, but never include the 9bp IR. The duplicated A enhancer core effectively substitutes for this deletion as evidenced by the stability of these rearrangements when virus is propagated in a variety of cell lines. A novel observation reported earlier (Couture, 1989) involves the junctions formed by the duplications and deletions in these pyPCC4 isolates. Nearly all duplication junctions, but no deletional junctions, form novel contiguous sequences that have near identity with the reported AP-1 core consensus sequence, 'TGACT(C/A)A'. It was recently reported that the duplication junction in a polyoma host range mutant with specificity for neuroblastoma cells generates a novel binding site for the NF-1 family of transcription factors (Caruso *et al.*, 1990).

The rearrangements found in pyPCC4 mutants are reflected to a lesser degree in several wild type variants of polyoma such as TOR and P16 (Ruley and Fried, 1983) and the more recently characterized strains LPT and ALB (Couture, 1989). Our goal has been to determine the functional rearrangements necessary for expression in PCC4 cells and their role in wild type viral strains. Specifically, we set out to determine whether the duplicated A enhancer domain was activating both replication and transcription in these polyoma variants or whether B enhancer elements for either of these functions have been retained such as the 9 bp IR. In order to address this question we present here our efforts to further distinguish and define the precise limits of the B enhancer cores for replication and transcription. The results from several replication experiments with laboratory enhancer rearrangements in PCC4 cells give new insight into the use of these methods for the study of viral tropism.

Results

Comparison of pyPCC4 mutants in the sequence of a novel isolate

A large plaque producing wild type isolate of the small plaque strain TOR was generously provided by Dr. Ian Macpherson. No

TABLE 1

SEQUENCE ANALYSIS OF DELETION AND DUPLICATION JUNCTIONS PRESENT IN WILD TYPE AND pyEC PCC4 HOST RANGE MUTANTS

AP-1: $^{5'}$TGACT $^{C}_{A}$ A$^{3'}$ $^{5'}$/T $^{T}_{G}$ AGTCA$^{3'}$

Strain	Duplication	Junction	Deletion	Junction
TOR	5139/5096	TG AGTCA	—	—
P16	5139/5096	TG AGTCA	—	—
LPT	5188/5096	TG AGTCA	5121/5266	AA TGTTT
	5139/5112	T GAGTCA		
pyPCC4-97	5187/5073	T GACTCT	5135/5220	GC ACCCA
pyPCC4-204	5187/5073	T GACTCT	5154/5251	TA TACTA
pyPCC4-A	5187/5073	T GACTCT	5121/5155	AA ATTAG
pyPCC4-5000	5193/5096	TT AGTCA	5123/5228	CT CTAGA
pyPCC4-500	5228/5103	CC TAAGC	5122/5250	AC TTACT
M206	5048/5100	CC AGTTA	5122/5229	AC TAGAA

The sequence of the non-palindromic AP-1 recognition site in both orientations is diagrammed at the top of that table. The reported duplication junctions of pyEC-PCC4-97, pyEC-PCC4-204 and pyEC-PCC4-A differ from the AP-1 consensus sequence at one terminal base; in all cases an A to T transition. Sequences are depicted in the same physical direction reading towards the origin of replication with a space representing the site of recombination (Vasseur *et al.*, 1980; Katinka *et al.*, 1981; Melin *et al.*, 1985b).

previously characterized unselected wild type strain has shown host range for EC cells. This TOR isolate (designated Large Plaque Toronto, LPT) was capable of expression and replication in PCC4 cells comparable to that reported for host range selected pyPCC4 isolates (Hales and Lehman, 1987). Isolation of previously described pyPCC4 host range mutants followed serial passage at high MOI of wild type viral stocks through PCC4. To help understand the role of enhancer rearrangements in pyPCC4 isolates we determined the sequence of this novel isolates enhancer region. Sequence analysis of the enhancer region of LPT reveals rearrangements similar, yet more extensive, to those characterized in previous pyPCC4 isolates (Fig. 1). As with these other isolates, redundancy of the A enhancer core is seen. Unlike previous isolates and the LPT parental strain TOR (Table 1) multiple complete and partial copies of this region have been generated. A characteristic deletion of the B enhancer is also present; in this case the deletion spans the entire B region. The 9bp IR has been retained in duplicate adjacent to both full A enhancer copies. While more extensively rearranged than previous pyPCC4 host range mutants, LPT maintains all features characteristic of this class of polyoma isolates.

The sequences of this and other pyPCC4 host range mutants were examined for common features not previously described. Specifically, the sequences formed at the deletion and duplication junctions were analyzed for the occurrence of consensus or other potentially non-random features. As shown in Table 1, eight of the ten duplications in these pyPCC4 isolates have generated near perfect core consensus sites ('TGACT(C/A)A') for the transcriptional activation factor AP-1 at their duplication junctions (Lee *et al.*, 1987). PEA1 is the murine homolog of the human transcriptional activation factor AP-1 and has been shown to interact with the polyoma A enhancer core in 3T3 cells (Piette and Yaniv, 1987; Martin *et al.*, 1988). The duplication endpoints map to various locations surrounding the A enhancer core suggesting a selection for this novel AP-1 site. A similar potential AP-1 site has also been generated in the wild type strains P16 and TOR. In contrast, no homologies, consensus, or recognizable factor binding sites occur at the junctions generated by the various deletions.

Origin proximal boundary of the B-enhancer element for replication

The two enhancer domains in polyoma contain sequences obligatory for both translational and transcriptional control of the viral life cycle. Our goal is to determine whether both of these functions were conferred on the duplicated A enhancer in these mutants or whether sequences from the B enhancer domain retained in all isolates, notably the 9 bp IR, performs one or both of these functions. In order to address this issue we generated and tested a number of unidirectional deletional mutants of the origin proximal B enhancer domain. These deletional constructs were tested for their ability to enhance transcriptional activity of linked polyomavirus early genes and to enhance replication of an adjacent polyomavirus origin of replication. To distinguish these two features and isolate the B enhancer core elements responsible for them, two series of constructs were established based upon these deletions. The two series differ only in the addition of contiguous early region coding sequences in the series pEOT-nt (where E, O, T and «nt» indicate the enhancer region, origin of replication, the polyoma early T antigen coding sequences and origin distal limit of the deletion respectively) (Fig. 2). We tested replication activity in the presence and absence of T antigen expression in PCC4-aza1 cells and the T antigen expressing cell line FOP.

Polyomavirus is unique in its requirement for an enhancer in the regulation of replication. As discussed earlier, the enhancer elements as currently defined are required for replication and appear to coincide closely with those for transcriptional activation. We further defined the origin proximal limit of the B enhancer element in FOP cells provided by Dr. J. Hassell. These cells constitutively express polyoma T antigens and therefore should allow replication of polyoma enhancer deletions that lack transcriptional enhancer elements but retain replication elements, if these two functions are separable. All enhancer constructs were tested in this permissive system for viability as measured by transient replication of transfected plasmids.

Replication of the pEO-nt series constructs in FOP cells

As can be seen in Fig. 3, when assayed in FOP cells, clones pEO-89 and pEO-215 were able to replicate to levels significantly higher than clones pEO-51, pEO-61, and pEO-66. The sharp drop in replication seen between the constructs pEO-89 and pEO-66 suggests violation of an element boundary by deletion of sequences from 5189 to 5166. When tested in several experiments, only very low levels of replication were observed for the remaining constructs in the pEO-nt series. The SV40 core consensus and PEA3 site of the B enhancer element core lie directly adjacent to nt 5189 yet the addition of these sequences up to nt 5215 (pEO-215) did not additionally contribute to replication enhancement. This finding indicates that sequences between 5166 and 5189 define a boundary for a minimal replication enhancer element in this cell system. It has been reported (Muller, 1988) that the B replication

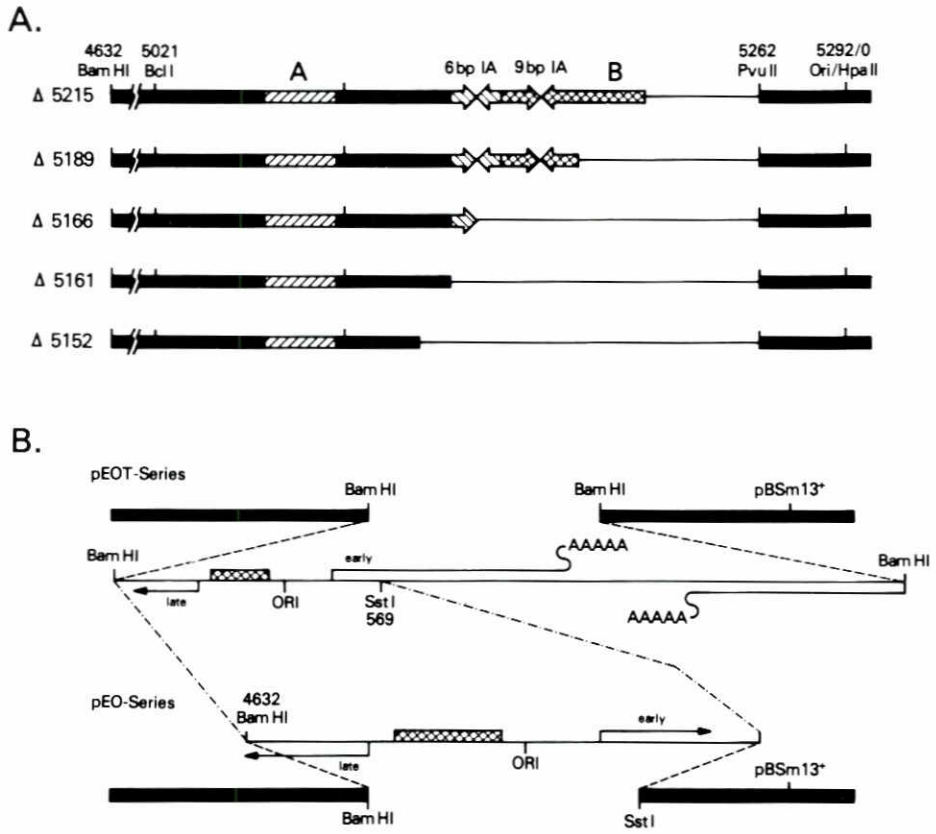

Fig. 2. Unidirectional deletional mutagenesis of the polyoma enhancer region. (A) *The Bam HI to Hpa II fragment (nt 4632-5292) was cloned into pBSm13+ (Stratagene). Unidirectional deletions from nt 5262 were created with exonuclease's III and VII as described in the text. 5' deletion endpoints are indicated.* **(B)** *Deleted enhancers were utilized as described to construct the pEO series containing early and late promoter sequences and viral origin of replication. pEOT constructs contain the addition of an intact early coding region.*

enhancer element lies between nt 5172-5202. Deletional analysis extending within this region was not reported. The results presented here suggest that the origin proximal endpoint of the B replication enhancer element may be more precisely positioned near nt 5189. The region contained within nt 5172-5189 is precisely the 9 bp inverted repeat which is conserved in pyPCC4 host range mutants.

Generation and replication of the pEOT-nt series in PCC4-azal cells

We compared the origin proximal limits of the replication enhancer in FOP cells and in PCC4 embryonal carcinoma cells. The PCC4 cell line used in this study does not constitutively express polyoma T antigens as does the FOP cell line and therefore polyoma replication is dependent on T antigen expression. The pEO-nt series described earlier was modified by the addition of the remainder of the polyoma coding region in order to generate intact viral genome-bearing plasmids. This new construct series, pEOT-nt, have an uninterrupted early coding region located correctly downstream of the enhancer region and are potentially able to transcribe early genes. The late coding region, however, is interrupted to prevent late gene expression and generation of viable virus in these transient assays.

The pEOT-nt series of constructs were assayed for their ability to replicate in PCC4 cells in a transient assay similar to that performed in FOP cells with the pEO-nt series (Fig. 4). Only construct pEOT-215 replicated in PCC4 cells to significant levels. This is in contrast to enhanced levels of replication also seen with the construct pEO-89 in FOP cells. In PCC4 cells, pEOT-89 replication dropped to levels comparable to that seen with pEOT-66. The data imply the presence of an element boundary located between nt 5189-5215 that is required for enhanced levels of replication in PCC4. It is important to consider, however, that the PCC4 replication assay is T antigen dependent while in FOP cells the assay is T antigen independent. It was possible that this additional sequence requirement is uniquely required for polyoma replication in PCC4 cells and does not reflect sequence requirements in permissive mouse cells. This possibility was assayed by testing several pEOT-nt constructs in NIH 3T6 cells. A strong replication signal was observed for the construct pEOT-215 and only a weak (albeit detectable) signal for the construct pEOT-89 (data not shown) comparable to the results obtained from PCC4 cells. The marked loss of signal intensity with the construct pEOT-89 in NIH 3T6 and PCC4 cells suggest that deletions extending into the region between nt 5189 and 5215 inactivate an element required ultimately for polyoma replication in both permissive and nonpermissive cell types.

Transcriptional activation dependence of pEO-89

It was necessary to determine whether the element described by deletions flanking nt 5189-5215 in PCC4 cells is a replication element not necessary in FOP cells, or if in fact it is a transcriptional element. It is possible that deletion of the region could prevent replication through the inability to drive T antigen expression, known to be required for replication in mouse fibroblasts. Additionally, it has been variously reported that polyoma replication in EC cells is either T antigen dependent or T antigen independent (Fujimura, and Linney, 1982; Trevor and Lehman, 1982). If in fact replication in

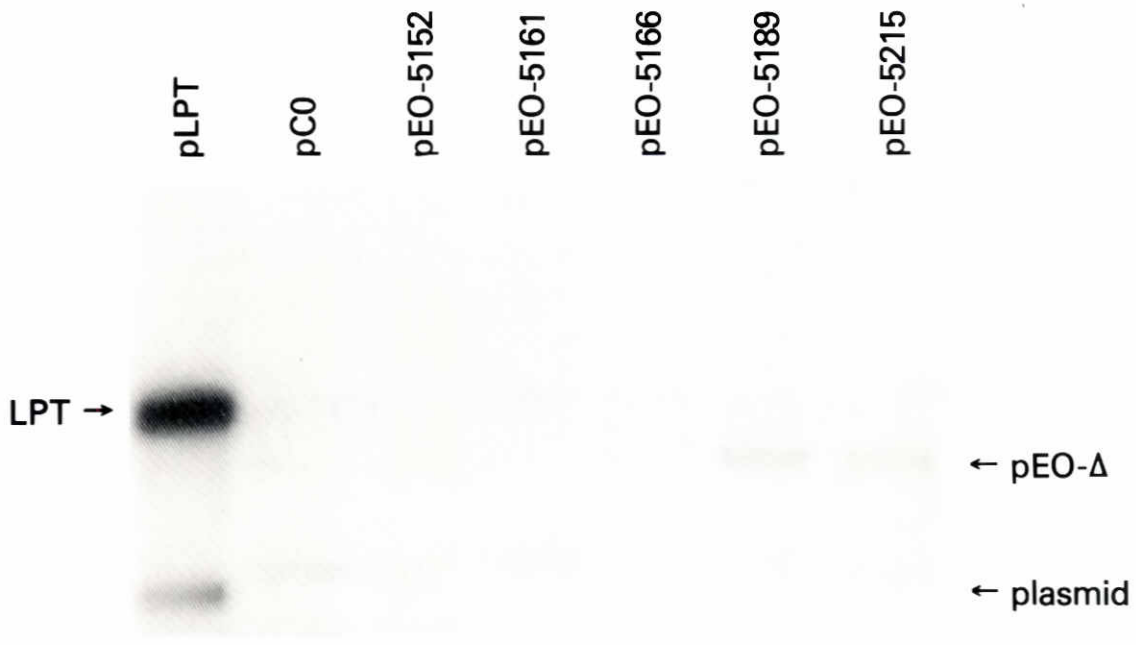

Fig. 3. Replication of the pEO-nt series in FOP cells. *Viral sequences were excised by digestion with Bam HI followed by digestion with Dpn I which exclusively recognizes unreplicated, methylated, input DNA. pC0 is a single A enhancer element construct similar to the pEO-series used as a control. Southern analyses were performed by hybridization to a wild type enhancer containing plasmid.*

PCC4 cells is T antigen independent this observation would indicate an additional sequence requirement for replication in these cells. The pEO-nt and pEOT-nt series were used in this assay to examine these questions of virus encoded factor dependency for replication in PCC4 cells. All of the pEO-nt constructs failed to replicate to significant levels in PCC4 cells (data not shown). Since only pEOT-215 had shown enhanced replication levels in PCC4 cells from above, replication activity would not have been predicted from pEO-52, -66 or -89. The loss of replication from pEO-215 in PCC4 cells supports the notion that replication, as demonstrated with pEOT-215, is T antigen dependent. To extend this finding, the replication activities of several pEO-nt series constructs were assayed in PCC4 cells for their ability to replicate when supplied in trans with T antigen in co-transfection experiments.

Trans-complementation of pEO-nt constructs in PCC4-aza1 cells

While the data presented above suggest that replication in PCC4 cells is T antigen dependent, it is possible that the lack of replication of pEO-nt based constructs is due to some unidentified requirement in these cells associated with transcriptional activation but not necessarily T antigen itself. To test this dependency on T antigen more directly, two pEO-nt constructs, pEO-66 and pEO-89, were co-transfected with pLPT, a pEOT-like construct containing the LPT enhancer region and capable of T antigen expression in PCC4 cells. If the loss of replication activity of pEOT-89 is due to the deletion of a transcription activation function leading to a loss of T antigen expression, then trans-supplied T antigen should reconstitute replication. In contrast, pEO-66 would not be expected to replicate even in the presence of T antigen, as was the case in FOP cells. In several experiments exemplified in Fig. 5, no replication was observed in control lanes when pEO-89 or pEO-66 were transfected alone as expected. When T antigen is supplied in trans by co-transfection with pLPT, pEO-89 but not pEO-66 was observed to replicate to significant levels. Replication of pEO-89 in contransfections is readily detected by the presence of a single additional band of intermediate mobility as indicated. The level of pEO-89+pLPT replication observed was not to very high levels yet was reproducible and enhanced from pEO-89 tranfected alone. The inability to drive high levels of replication in co-transfection experiments may be due to competition for limiting factors in the cell. This effect has been observed by Mueller *et al.* (1988) to explain similar results in earlier reports. Alternatively, it might be assumed that the increase may be due to fortuitous competition for negative acting factors by pLPT. This is not likely, however, as total DNA concentrations were equal, and in some experiments lower, than those used in experiments with pEOT-89 alone. It is concluded that the addition of T antigen-expressing constructs allows for enhanced levels of replication from pEO-nt constructs in a trans-acting manner in PCC4 cells. This supports the hypothesis that an element which is defined, in part, by sequences between nt 5189-5215 is critical for transcriptional activation of polyoma but is not required for viral replication. This data also supports our laboratory's earlier report that polyoma replication is T antigen dependent in PCC4 cells.

Discussion

The previously reported generation of polyoma virus mutants with host range restriction for the EC cell line PCC4 required serial passage through PCC4 cells of wild type viral stocks at high MOI. The wild type isolate, LPT, described here was obtained without selection for EC restriction yet expresses in PCC4 cells to levels comparable to those reported for other pyPCC4 isolates. Similar to

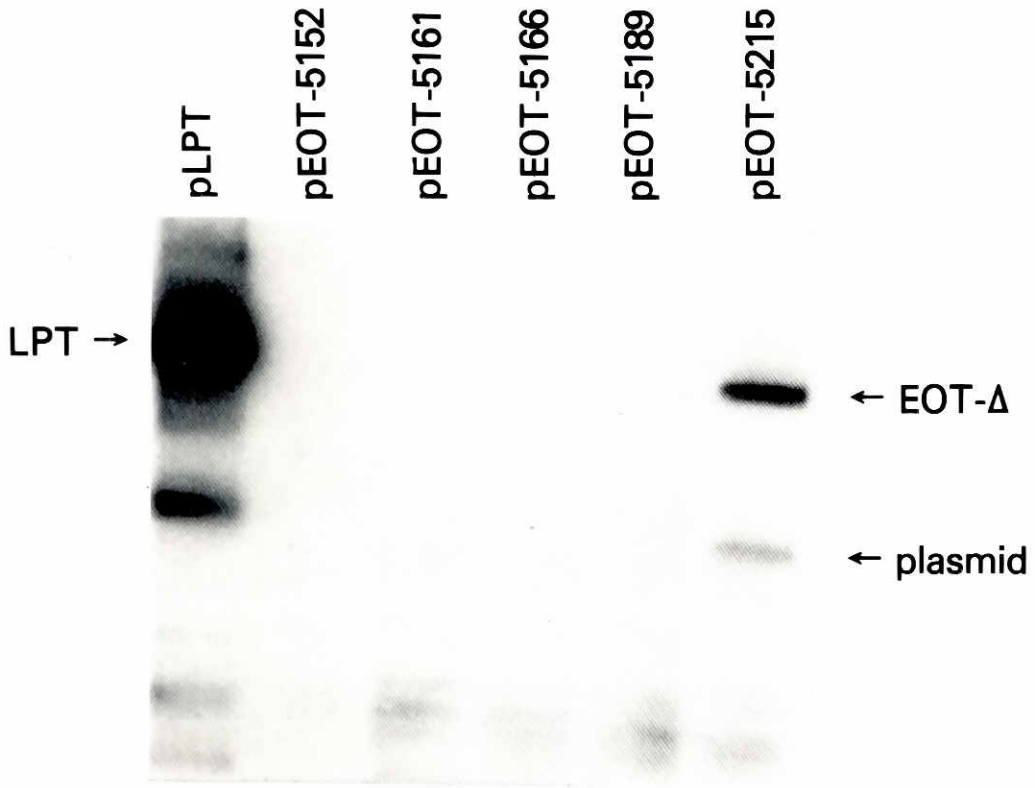

Fig. 4. Replication of the pEOT-nt series in PCC4 cells. *Southern blot analysis of Dpn I and Bam HI digested Hirt DNA from transfected PCC4 cells. As described in Fig. 3. Viral and plasmid sequences are indicated.*

pyPCC4 isolates, the enhancer region rearrangements of LPT confer the altered host range restriction (data not shown) and parallel those observed in all pyPCC4 isolates. This suggests that passage of polyoma through PCC4 cells selects for rearrangements that may be preexisting in wild type stocks. This is reflected in the isolation of several wild type polyoma strains with similar (albeit less extensive) enhancer rearrangements such as P16, TOR and ALB.

Comparison of pyPCC4 and wild type isolates with enhancer rearrangements reveals that in addition to sharing a common duplication of the A enhancer core, most isolates have generated a novel AP-1 core consensus site. Of significance is that this site does not arise from utilization of common duplication endpoints suggesting a functional selection for this sequence. Recent evidence with a neuroblastomatropic mutant of polyoma (Caruso *et al.*, 1990) supports the contention that these novel transcription factor sites may be functional.

P16, with a duplicated A enhancer core and lacking any other features common to pyPCC4 mutants demonstrates a tropism for PCC4 cells intermediate to that of the non-expressing strains A2 and A3 and that of LPT (Table 2). It is possible that this is facilitated by the novel AP-1 site or by the redundancy of other duplicated factor binding sites such as PEA2 and PEA3. The observation that no similar sites are generated at the deletion junctions of pyPCC4 isolates suggests that generation of a novel AP-1 site is not a function of the recombinant machinery used by polyoma. Because similar duplication in the absence of any deletion exists in wild type polyoma strains with only limited tropism for PCC4 cells, and since a deletion without a concomitant duplication would result in a virus with a single enhancer element and reduced growth potential, we conclude that the deletion necessarily follows the duplication event.

If the duplication arises to augment transcription via generation of novel AP-1, then what is the purpose of the deletions found in all pyPCC4 isolates? The deletions might occur to offset genome size constraints or to remove deleterious, possibly suppressor, sequences. The argument that the deletions arise to compensate for

TABLE 2

PERCENTAGE OF PCC4-AZA1 CELLS EXPRESSING T ANTIGEN AND V ANTIGEN FOLLOWING INFECTION WITH WILD TYPE POLYOMAVIRUS

		Days Post Infection		
		1	3	5
Virus Strain				
A2	T	0	<0.1	<0.1
	V	0	0	0
A3	T	0	<0.1	<0.1
	V	0	0	<0.1
LPT	T	<5	10-30	>50
	V	<1	<5	<50
P16	T	<0.1	<1	<5
	V	n.d.	n.d.	n.d.

The cells were fixed and stained with polyclonal antibody to polyoma T antigen (T) and the viral coat proteins (V). nd = not done.

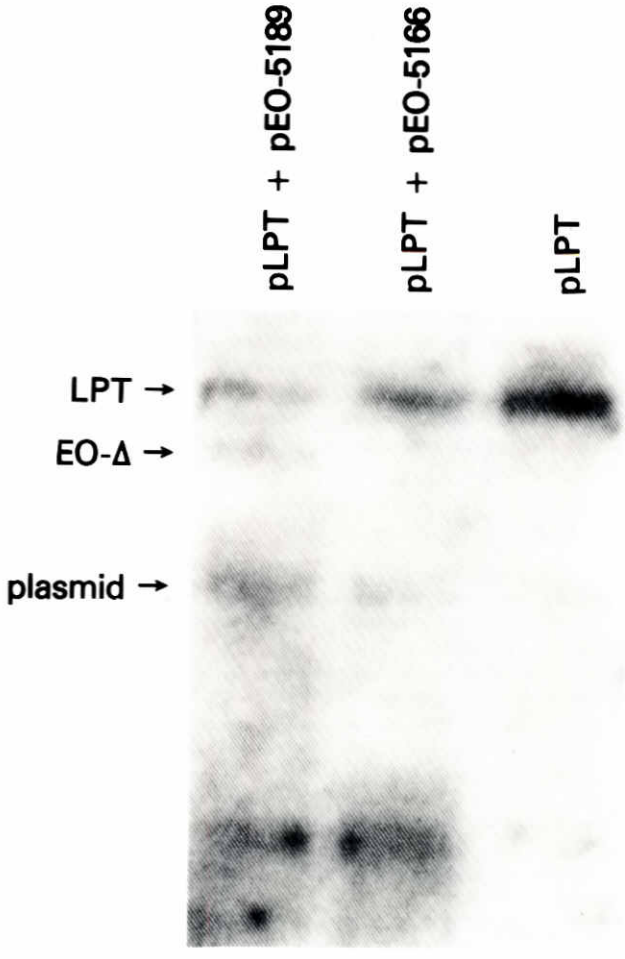

Fig. 5. Replication assay of pEO-5189 and pEO-5166 when co-transfected with pLPT into PCC4 cells. *As described in Fig. 3. Replicated and excised full length LPT is indicated as are intermediate molecular weight viral sequences from the PEO-nt plasmids and the remaining plasmid sequences.*

an increased viral genome is likely not to be entirely valid. The enhancer region of the strain LPT is larger by 79 base pairs than the wild type strain A2, yet has remained stable since 1972 and was, significantly, isolated as a large plaque isolate of a small plaque parent strain. That no common sequences are deleted in pyPCC4 isolates would strongly argue against removal of specific suppressor sequences in this region. No pyPCC4 isolate has been reported, however, that does not contain a deletion of some portion of the B enhancer region underscoring the potential importance for this deletion. It is possible that the deletion allows for a necessary repositioning of the A enhancer core with respect to other polyoma elements such as the replication origin.

To address the functional role of the rearrangements in pyPCC4 isolates it is first necessary to discriminate and delineate precisely the boundaries of the B enhancer element for replication and transcription in PCC4 cells. Experiments described in the permissive FOP cell line and in PCC4-aza1 confirm that the origin proximal boundary for the B transcriptional enhancer element lies at or near nt 5215 and that the replication B enhancer element lies at or near nt 5189. The replication B element coincides with a 9 bp inverted repeat that, interestingly, is never deleted in pyPCC4 isolates. These results are in contrast to those reported by Rochford *et al.* (1990), who did not observe a difference in replication activity between the wild type polyoma enhancer region and a deletion leaving only a single A enhancer (deletion of nt 5132-5169). That group did not assay intermediate B enhancer deletions and the difference in relative activities may reflect a difference in sensitivity of the assay in our hands.

The observed level of replication with construct pEOT-5215 in PCC4 cells was unexpected. Duplication of the A enhancer core with a more extensive deletion had been predicted as minimally necessary for enhanced replication. That an essentially intact polyoma enhancer region with no rearrangements could replicate in PCC4 cells required the testing of the integrity of the PCC4-aza1 cell line. Repeated infections of these cells with various polyoma wild type strains established that the PCC4-aza1 seed lot used in these experiments was refractory to polyoma expression as measured by T antigen immunofluorescent staining (Table 2). Replication assays performed on PCC4 cells transfected with pEOT-like plasmid clones of the wild type polyoma strains A2, A3 and P16. All clones replicated to levels seen with the pyPCC4 host range pEOT-nt clone, pLTP (data not shown). This led us to conclude that transfected polyoma DNA was not susceptible to the block observed when virus was introduced as infectious particles. Constructs that replicated in FOP cells or NIH 3T6 cells were comparably competent in PCC4 cells. In contrast, transfected polyoma DNA is not unregulated in that T antigen remains necessary for replication in PCC4 cells. The reason for this permissivity to replication of transfected DNA in the absence of significant T antigen expression is not clear. It is possible that levels of T antigen not detectable by immunofluorescence are present and sufficient for replication. Several other series were tested in PCC4 cells based upon the original deletions described here. These include constructs with duplicated A enhancer cores, additional AP-1 binding sites, and constructs meant to test positional constraints on enhancer elements. These constructs were found to replicate equally in PCC4-azal and FOP (data not shown) as expected for any polyoma enhancer construct with intact A and B enhancer core elements tested in permissive cells. The loss of the block in PCC4 cells to replication of transfected polyoma DNA makes future use of a plasmid/viral construct-based model for analysis of the functional roles of rearrangements in pyPCC4 mutants questionable. Infectious polyoma clones will need to be constructed to determine the functional role of these rearrangements.

Materials and Methods

Cell culture

FOP 5b cells were generously provided by Dr. J. Hassell, McGill University and the PCC4-aza1 was obtained from Dr. Wendell Speers and is a varient clone of the azacytadine resistant line PCC4-aza (Jacob *et al.*, 1973). PCC4-aza1 cells and FOP 5b cells were maintained in Minimal Eagles Medium supplemented with 10% fetal bovine serum and antibiotics at 37°C with 5% CO_2.

Viral strains

The polyoma strain P16 was generously provided by T. Benjamin. The TOR isolate, LPT, had been provided to us in 1972 by Ian Macpherson. An aliquot of the original stock was used for these experiments.

Construction of recombinant plasmids

The wild type polyoma strains A2, A3, ALB, and P16 were cloned into the plasmid pBSm13+ (Stratagene) at the Bam HI site. ALB is an isolate of the A3 strain containing a 25 bp direct repeat of the sequences 5113-5137 that includes the PEA1 and PEA2 binding sites in the A enhancer core. The numbering system of Tyndall *et al.* (1981) is used throughout this paper.

The recombinant enhancer constructs are based on a series of unidirectional deletional mutants derived from the ALB strain with the 25 bp duplication deleted. The Bam HI to Hpa II (nt 4632 to 5292) fragment was cloned into the Bam HI and Sma I sites of pBSm13+. The ensuing plasmid was opened with Sst I and Eco RI and digested with Exo III and Exo VII as previously described (Yanisch-Perron *et al.*, 1985). The 25 bp repeat of the ALB strain was removed from each of these constructs by digestion with Bam HI, Pvu II and Sst I (the Pvu II site at nt 5128 in ALB is duplicated), and ligation of the appropriate fragments into pBSm13+ at the Bam HI and Sst I sites. Both series were digested with Eco RI, blunt ended, then Bam HI digestion followed by ligation into the Bam HI and Hinc II sites of pBSm13+.

This series was further modified by addition of origin and early promoter sequences from the ALB strain (identical with A3) from Pvu II to Sst I (nt 5262-569) cloned into the Sst I to Hinc II sites of pBSm13+. Digestion of this origin containing plasmid and each unidirectional deletion isolates with Pst I and either Sst I or Bam HI respectively followed by ligation into pBSm13+ yielded the series pEO-nt (where E, O and «nt» indicate the enhancer region, origin of replication and origin distal limit of the deletion respectively). These constructs were expanded to the pEOT-nt series (where T represents the polyoma early T antigen coding sequences) by incorporation of the remaining genomic sequences of A3 by digestion with Hind III (a multiple cloning region site) and Bgl I (nt 87) and ligation with the large Bgl I to Bam HI fragment of A3 and a Hind III and Bam HI digested PBSM13+. Constructs were amplified in JM109 and 0.5 liter alkaline lysis preparations were obtained and purified by passage through mini purification columns (Five Prime-Three Prime). Constructs were confirmed by dideoxy chain termination sequencing (Sanger *et al.*, 1977).

DNA transfections and replication assay

Recombinant plasmids were transfected into cells according to Chen and Okayama (1987) with the following modifications. FOP (Rochford *et al.*, 1990) and PCC4-aza1 cells at $3x10^5$ and $1.5x10^5$ cells respectively per 10cm culture dish were plated 24 h prior to transfection. Duplicate plates were transfected with 1 to 8 µg plasmid DNA with 15 µg sheared salmon sperm DNA at 37°C with 3% CO_2 for 12 h (FOP) or 10 h (PCC4-aza1). Plates were washed twice with phosphate buffered saline and media were replenished.

Hirt (1967) extracts were performed 48 h after transfection on FOP cells and PCC4-aza1 cultures and replicated plasmid DNA was measured as previously described (Muller *et al.*, 1988) with the following modifications. Low molecular weight DNA was digested with Dpn I and Bam HI and separated on a 1% agarose gel, transferred to GeneScreen, and hybridized to ^{32}P-labeled, random primed pBSm13+ (Stratagene) plasmid containing the entire A3 strain genome. Blots were exposed to Kodak XAR-5 film (Eastman Kodak Co.) for 2 to 24 h. Replication experiments were repeated in duplicate at least twice.

Immunohistochemical staining for T antigens

Detection of polyoma T antigen in transfected or infected PCC4 cells was as previously described (Hales and Lehman, 1987).

Acknowledgments

When I (JML) was considering a postdoctoral fellowship in 1970 I was fascinated with the fact that certain tumors, as exemplified by the teratocarcinoma, were able to remove the stem cells from the tumor population following differentiation. This interesting tumor model system had been defined by Drs. G. Barry Pierce and Leroy Stevens. Therefore, after my arrival in Colorado my interaction with Dr. Pierce and his laboratory developed through a mutual interest in experimental pathology and tumor biology. I found my research focusing on issues in tumor biology related to the murine teratocarcinoma. Barry demonstrated an interest and willingness to collaborate, and offered advice and suggestions in pursuit of our experimental problems. This may be one of the reasons why he offered me a faculty position as an Instructor, and then Assistant Professor, Associate Professor, and Professor at the University of Colorado School of Medicine. I remember, with joy, those days in the Pathology Department where the faculty, both clinical and basic scientists, were able to interact and gain mutual benefit from these interactions. I look with pride upon these years that allowed me to develop my research program and independence in an environment that nurtured scientists and clinicians. Much of this was due to the environment created by Dr. G. Barry Pierce. In 1985 I left the Department of Pathology at the University of Colorado to become Chairman of the Department of Microbiology, Immunology and Molecular Genetics at the Albany Medical College, thus completing a cycle of Westward migration to Denver and then a return to the Northeast. I look back with pride on the training and development that occurred during my years at Colorado in education, research and administration which has formed a significant basis for my continuing career.

This work was supported by grant CA41608 from the National Institutes of Health. The authors wish to thank Ms. Jo Ann D'Annibale for preparation of this manuscript.

References

CARUSO, M., LACOBINI, C., PASSANANTI, C., FELSANI, A. and AMATI, P. (1990). Protein recognition sites in polyomavirus enhancer: formation of a novel site for NF-1 factor in an enhancer mutant and characterization of a site in the enhancer D domain. *EMBO J. 9:* 947-955.

CHEN, C. and OKAYAMA, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol. 7:* 2745-2752.

COUTURE, L.A. (1989). Enhancer requirements of polyomavirus for expression in embryonal carcinoma cells. Thesis, Albany Medical College.

DE VILLIERS, J. and SCHAFFNER, W.A. (1981). Small segment of polyoma virus DNA enhances the expression of a cloned B-globin gene over a distance of 1400 base pairs. *Nucleic Acids Res. 23:* 6251-6264.

DE VILLIERS, J., OLSON, L., TYNDALL, C. and SCHAFFNER, W. (1982). Transcriptional 'enhancers' from SV40 and polyoma virus show a cell type preference. *Nucleic Acids Res. 10(24):* 7965-7976.

FUJIMURA, F.K. and LINNEY, E. (1982). Polyoma mutants that productively infect F9 embryonal carcinoma cells do not rescue wild-type polyoma in F9 cells. *Cell 79:* 1479-1483.

FUJIMURA, F.K., DEININGER, P.L., FRIEDMAN, T. and LINNEY, E. (1981). Mutation near the polyoma DNA replication origin permits productive infection of F9 embryonal carcinoma cells. *Cell 23:* 809-814.

HALES, K. and LEHMAN, J.M. (1987). Further characterization of the interaction of polyoma virus and Simian virus 40 with embryonal carcinoma cells. *Exp. Mol. Pathol. 47:* 48-58.

HERBOMEL, P., BOURACHOT, B., and YANIV, M. (1984). Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. *Cell 39:* 653-662.

HIRT, B. (1967). Selective extraction of polyoma DNA from infected mouse cultures. *J. Mol. Biol. 26:* 365-369.

JACOB, H., BOON, T., GAILLARD, K., NICHOLAS, J.F. and JACOB, F. (1973). Teratocurinome de la souris: I solement, culture et propietas de cellules a potentialities multiples. *Ann. Microbiol. 124B:* 269-274.

KATINKA, M., VASSEUR, M., MONTREAU, N., YANIV, M. and BLANGY, D. (1981). Polyoma DNA sequences involved in control of viral gene expression in murine embryonal carcinoma cells. *Nature 290:* 720-722.

KLEINSMITH, L.J. and PIERCE, G.B. (1964). Multipotentiality of single embryonal carcinoma cells. *Cancer Res. 24:* 1544-1551.

LEE, W., HASLINGER, A., KARIN, M. and TJIAN, R. (1987). Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. *Nature 325:* 368-372.

LEHMAN, J.M., SPEERS, W.C., SWARTZENDRUBER, D.E. and PIERCE, G.B. (1974). Neoplastic differentiation: characteristics of cell lines derived from a murine teratocarcinoma. *J. Cell. Physiol. 84:* 13-28.

MARTIN, M.E., PIETTE, J., YANIV, M., TANG, W-J. and FOLK, W.R. (1988). Activation of the polyomavirus enhancer by a murine activator protein 1 (AP1) homolog and two contiguous proteins. *Proc. Natl. Acad. Sci. USA 85:* 5839-5843.

MELIN, F., PINON, H., KRESS, C. and BLANGY, D. (1985a). Isolation of polyomavirus mutants multiadapted to murine embryonal carcinoma cells. *J. Virol. 53:* 862-866.

MELIN, F., PINON, H., REISS, C., KRESS, C., MONTREAU, N. and BLANGY, D. (1985b). Common features of polyomavirus mutants selected on PCC4 embryonal carcinoma cells. *EMBO J. 4:* 1799-1803.

MUELLER, C.R., MULLER, W.J. and HASSELL, J.A. (1988). The polyomavirus enhancer comprises multiple functional elements. *J. Virol. 62:* 1667-1678.

MULLER, W.J., DUFORT, D. and HASSELL, J.A. (1988). Multiple subelements within the polyoma enhancer function synergistically to activate DNA replication. *Mol. Cell. Biol. 8:* 5000-5015.

PIERCE, G.B. (1967). Teratocarcinoma: model for a developmental concept of cancer. In *Current Topics in Developmental Biology 2* (Eds. A.A. Moscona and A. Mongony). Academic Press, NY, pp. 223-246.

PIETTE, J. and YANIV, M. (1987). Two different factors bind to the a-domain of the polyoma virus enhancer, one of which also interacts with the SV40 and c-fos enhancers. *EMBO J. 6:* 1331-1337.

ROCHFORD, R., DAVIS, C.T., YOSHIMOTO, K.K. and VILLARREAL, L.P. (1990). Minimal subenhancer requirements for high-level polyoma virus DNA replication: a cell-specific synergy of PEA3 and PEA1 sites. *Mol. Cell. Biol. 10:* 4996-5001.

RULEY, H.E. and FRIED, M. (1983). Sequence repeats in a polyoma virus DNA region important for gene expression. *J. Virol. 47:* 233-237.

SANGER, F., NICKLEN, S. and COULSON, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA 7:* 5463-5467.

SWARTZENDRUBER, D.E. and LEHMAN, J.M. (1975). Neoplastic differentiation: interaction of Simian virus 40 and polyoma virus with murine teratocarcinoma cells. *J. Cell. Physiol. 85:* 179-187.

SWARTZENDRUBER, D.E., FRIEDRICH, T.D. and LEHMAN, J.M. (1977). Resistance of teratocarcinoma stem cells to infection with Simian virus 40: early events. *J. Cell. Physiol. 93:* 25-30.

TREVOR, K. and LEHMAN, J.M. (1982). The interaction of polyoma virus with F9 embryonal carcinoma cells and chemically induced differentiated progeny: fate of the viral DNA and expression of viral antigens. *J. Cell. Physiol. 2:* 69-83.

TYNDALL, C., LAMANTIA, G., THACKER, C.M., FAVALORO, J. and KAMEN, R. (1981). A region of the polyoma virus genome between the replication origin and late protein coding sequences is required in cis for both early gene expression and viral DNA replication. *Nucleic Acids Res. 9:* 6231-6250.

VASSEUR, M., KRESS, C., MONTREAU, N. and BLANGY, D. (1980). Isolation and characterization of polyoma virus mutants able to develop in embryonal carcinoma cells. *Proc. Natl. Acad. Sci. USA 77:* 1068-1072.

VELDMAN, G.M., LUPTON, S. and KAMEN, R. (1985). Polyomavirus enhancer contains multiple redundant sequence elements that activate both DNA replication and gene expression. *Mol. Cell. Biol. 5:* 649-658.

WEIHER, H., KONIG, M. and GRUSS, P. (1982). Multiple point mutations affecting the simian virus 40 enhancer. *Science 219:* 626-631.

YANISCH-PERRON, C., VIEIRA, J. and MESSING, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene 33:* 103-119.